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Hakija
Applicant

1. Norlund, Henri Rainer, Jyväskylä
2. Laitinen, Olli Heikki, Kuopio
3. Hytönen, Vesa Pekka, Jyväskylä
4. Kulomaa, Markku Sakari, Siivikkala

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Osoite: Arkadiankatu 6 A
P.O.Box 1160
FIN-00101 Helsinki, FINLAND

Puhelin: 09 6939 500
Telephone: + 358 9 6939 500

Telefax: 09 6939 5328
Telefax: + 358 9 6939 5328

Avidin mutants

FIELD OF THE INVENTION

The present invention is in the field of modified avidin, and more particularly in the area of avidin fusion proteins wherein two circularly permuted avidin monomers
 5 were fused to a dual-chain avidin (dcAvd) leading to a dual-chain pseudo-tetrameric avidin containing two polypeptides instead of four in the native protein. The present invention provides further a single-chain avidin (scAvd) where two dcAvd-molecules are fused together via a linker to form a single polypeptide with four binding sites for biotin.

10 BACKGROUND OF THE INVENTION

Avidin, a glycoprotein found in chicken egg white as well as its distant relative, streptavidin from *Streptomyces* bacteria (Argarana, C.E. et al. *Nucleic Acids Res* **14**, 1871-82 (1986)), have high affinity for biotin. This firm interaction has been utilized in countless applications in the different fields of life sciences to probe,
 15 label and affinity separate biomolecules. Another premise for the system has been the easy coupling of biotin to almost any other molecules without compromising the strong (strept)avidin-biotin bond or the function of the coupled molecule (Green, M.N. *Advances in Protein Chemistry* **29**, 85-133 (1975), Wilchek, M. & Bayer, E.A. *Biomol Eng* **16**, 1-4 (1999)).

Avidin is a homotetramer encoded by a single gene (Ahlroth, M.K. et al. *Anim Genet* **31**, 367-75 (2000), Wallen, M.J. et al. *Gene* **161**, 205-9 (1995)). This fact together with the almost perfect 222 point structural symmetry (Pugliese, L. et al. *Journal of Molecular Biology* **231**, 698-710 (1993)) and the orientation of the subunits in the protein guarantees that all four biotin-binding sites in avidin have
 25 equally high affinity towards biotin. From the evolutionary point of view this also means that in the protein all four binding sites co-evolve. The monomers of avidin are simple classical up-and-down β -barrel proteins. They have identical topology of eight β -strands and their interconnecting loops (Pugliese, L. et al. *Journal of Molecular Biology* **231**, 698-710 (1993), Hendrickson, W.A. et al. *Proceedings of The National Academy of Sciences of the United States of America* **86**, 2190-2194 (1989), Livnah, O. et al. *Proceedings of The National Academy of Sciences of the United States of America* **90**, 5076-5080 (1993)). If the 3-D structure of avidin is superimposed with that of streptavidin it is evident that the termini and also the
 30

- biotin-binding pocket situates in topologically analogous regions in these proteins. Two monomer pairs in each avidin tetramer share a large common interface and they are therefore called as the structural dimers. The complete tetramer is composed of two of such structural dimers (Pugliese, L. et al. *Journal of Molecular Biology* 231, 698-710 (1993), Hendrickson, W.A. et al. *Proceedings of The National Academy of Sciences of the United States of America* 86, 2190-2194 (1989), Livnah, O. et al. *Proceedings of The National Academy of Sciences of the United States of America* 90, 5076-5080 (1993), Weber, P.C. et al. *Science* 243, 85-88 (1989)).
- During the recent years avidin and streptavidin have been engineered via site-directed mutagenesis (Laitinen, O.H. et al. *J Biol Chem* 276, 8219-24 (2001), Laitinen, O.H. et al. *FEBS Lett* 461, 52-8 (1999), Marttila, A.T. et al. *FEBS Lett* 441, 313-7 (1998), Marttila, A.T. et al. *FEBS Lett* 467, 31-6 (2000), Reznik, G.O. et al. *Proceedings of The National Academy of Sciences of the United States of America* 95, 13525-30 (1998), Reznik, G.O. et al. *Nat Biotechnol* 14, 1007-11 (1996), Sano, T. et al. *Proceedings of The National Academy of Sciences of the United States of America* 94, 6153-6158 (1997), Sano, T. & Cantor, C.R. *Proceedings of The National Academy of Sciences of the United States of America* 92, 3180-3184 (1995), Chilkoti, A. et al. *Bio/Technology* 13, 1198-1204 (1995), Chilkoti, A. et al. *Proceedings of The National Academy of Sciences of the United States of America* 92, 1754-1758 (1995). Chu, V. et al. *Protein Sci* 7, 848-59 (1998), Freitag, S. et al. *Proceedings of The National Academy of Sciences of the United States of America* 96, 8384-9 (1999), McDevitt, T.C. et al. *Biotechnol Prog* 15, 391-6 (1999)). In some studies the focus has been on the adjustment of the physico-chemical properties of (strept)avidin whereas in other studies the target has been on the fine-tuning of the biotin-binding affinity. Nevertheless, as these mutant protein subunits are single gene products the desired changes, produced by mutations, take effect in all (strept)avidin subunits concurrently.
- In several cases, however, it would be beneficial to alter for example the binding-affinity only in some subunits of the tetramer while conserving the tight binding in the rest of the binding sites. Chilkoti et al. have introduced a partial solution to this problem by producing separately two streptavidin forms, one having natural high-affinity biotin-binding capacity and another with reduced affinity (Chilkoti, A. et al. *Bio/Technology* 13, 1198-1204 (1995)). They denatured these two forms and mixed them, after which the mixture was renatured. Nonetheless, the refolding led

to many alternative forms: some of them contained four high affinity binding sites whereas other forms had a raising series of lower affinity binding sites, finally ending in the form that contained four lower affinity binding sites. In this sense the genetic fusion of the subunits might be more straightforward and effective strategy to create (strept)avidin molecules containing biotin-binding sites of variable affinity because it would lead into the uncoupling of the monomers as an evolutionary and protein engineering unit and therefore production of better quality preparations. The N- and C-termini of the distinct (strept)avidin subunits are, however, situated far away from each other in the quaternary structure and therefore it is presumable that any simple fusion strategy would fail.

A common approach to study protein folding and significance of secondary structure topology to the protein structure and function is the creation of circularly permuted forms of the examined proteins (Uliel, S. et al. *Protein Eng* 14, 533-542 (2001)). Creation of circular permutations is an approach wherein the normal termini of a polypeptide are linked and new termini are created by breaking the backbone elsewhere. In many polypeptides, the normal termini are in close proximity and can be joined by a short amino acid sequence. The break in the polypeptide backbone can be at any point, preferably at a point where the natural function and folding of the polypeptide are not destroyed. In most cases proteins stand the circular permutations considerably well without exhibiting radical alterations in their structures or functions. Following circular permutation new C- and N-termini are created to the protein, allowing creation of fusion proteins wherein the fused peptide or protein is attached at a different place on the host protein.

Usually in this approach the original N- and C-termini are joined together with a linker peptide whereas the new termini are typically introduced into loop regions. Chu et al. have described a circularly permuted streptavidin that showed almost identical 3-D structure when compared to that of the native protein (Chu, V. et al. *Protein Sci* 7, 848-59 (1998)). In that study the circular permutation strategy was used as a tool to delete the loop between β -strands three and four in streptavidin monomers. This loop is functionally important because it undergoes the open to closed conformational change upon biotin binding (Hendrickson, W.A. et al. *Proceedings of The National Academy of Sciences of the United States of America* 86, 2190-2194 (1989), Weber, P.C. et al. *Science* 243, 85-88 (1989)). Consequently, when the new termini were introduced in this loop, the affinity of the resultant

mutant for biotin collapsed six orders of magnitude as compared to the wild-type (wt) streptavidin.

In US patent 6492492 circularly permuted streptavidins were designed by altering one monomer but the monomers were not fused together. The resulting streptavidin has four monomers as the wt protein.

BRIEF SUMMARY OF THE INVENTION

The objective of the present invention is to create a dual-chain mutant avidin with four binding sites that would consist of only two polypeptide chains. Another objective of the present invention is to provide a single-chain avidin where two dual-chain avidin molecules are fused together to form a single polypeptide with four binding sites for biotin.

Two circularly permuted chicken avidin monomers were designed in aim to fuse them and produce a fusion avidin containing two polypeptides instead of four in the native protein. First, two circularly permuted avidin monomers (cpAvd5→4 and cpAvd6→5) were constructed, wherein the new termini were in an ideal position to allow the building of the desired genetic fusion. The construction of this dual-chain avidin (dcAvd) by fusing the monomers of the structural dimer together is described and it is shown that it forms wild type (wt) like pseudo-tetrameric quaternary structure and that its four biotin-binding sites exhibit high-affinity biotin binding. Therefore this construct is useful as a structural scaffold to change the affinity parameters in some subunits while preserving the high biotin-binding affinity in the remaining binding sites. The circularly permuted avidin monomers (cpAvd5→4 and cpAvd6→5) could be further mutagenized by site specific mutagenesis before fusion.

In single-chain avidin two dual-chain avidin molecules are fused together via a linker to form a single polypeptide with four binding sites for biotin. Each of the four binding sites can be modified by protein engineering independently.

In the circularly permuted avidins the natural termini of the polypeptide monomer are joined and the protein is opened at another point to create new C- and N-termini. The changed locations of the new N- and C-termini in the circularly permuted avidin monomer enable the creation of the fusion construct in which a short spacer peptide joins the two subunits. The original N- and C-termini of the wild-type avidin are connected to each other via glycine/serine rich linker. The

circularly permuted avidin monomers (cpAvd5→4 and cpAvd6→5) are fused and the resulting fusion peptides (dcAvd) form a pseudo-tetrameric dual-chain avidin, which is biologically active in the sense of biotin binding and shows similar structural characteristics as wild-type avidin. The dcAvd described in the present study makes the development of dual-affinity avidins possible by allowing the adjustment of the ligand binding properties in the half of the binding sites while enabling the conserved affinity in the rest of the sites.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. (A) The amino acid sequence of the two fused monomers of avidin (dcAvd) (SEQ ID NO:2) with the locations of the wt β -strands indicated. The underlined ARK denotes the three first amino acids of wt avidin (SEQ ID NO:1). The artificial linkers GGSGGS (SEQ ID NO:3) and the monomer-monomer transition spacer SGG are highlighted with boxes. The part that is derived from cpAvd5→4 is underlined with the red bar and the part derived from cpAvd6→5 is underlined with the blue bar. (B) Schematic illustration of the fused monomers of the wt structural dimer in dcAvd. The artificial linkers (GGSGGS) that connect the original termini and the intermonomeric spacer (SGG) are circled. The left part in red is derived from cpAvd5→4 and the right part in blue is derived from cpAvd6→5.

Figure 2. Denaturing SDS-PAGE analysis confirmed that the size of the fused monomers present in the dcAvd is twice of that compared to the size of the circularly permuted avidin monomers cpAvd5→4 (cp54) and cpAvd6→5 (cp65). The unit of the molecular weight (MW) is kDa.

Figure 3. Sensitivity of the mutants to proteinase K treatment. The values indicate the percentage of intact monomer present in the sample after 30 min (1) 60 min (2) and 16 hours (3) treatment. The samples are (A) cpAvd5→5, (B) cpAvd6→5, (C) dcAvd and (D) wt avidin.

Figure 4. The quenching of biotin-coupled BF560 by avidin. The spectra of 50 nM biotin-Bf560 solution in 50 mM Na-phosphate containing 650 mM NaCl was measured (red). Chicken avidin (Belovo S. A., Bastogne, Belgium) was added to final subunit concentration of 83 nM and green spectra was obtained.

Figure 5. The dissociation of biotin-coupled fluorescent probe (ArcDia BF560) with excess unlabeled biotin from proteins analyzed in this example. Avd(Y33H)

(Marttila, A. T. et al. *Biochem J.* 369, 249-254 (2003)) shows rapid and complete dissociation of the fluorescent biotin probe from the four identically mutagenised (Y33H) subunits of tetramer (black). p54(I117C)+p65, instead, showed slow dissociation of the fluorescent biotin probe (green), comparable to that of wild-type
 5 avidin (not shown). Only half of the binding sites of p54(I117C)+p65(Y33H) have the mutation, and approximately half of the bound biotinylated probe is released within one hour (red).

Figure 6. DNA sequence which codes for scAvd of SEQ ID NO: 24. For said DNA sequence also SEQ ID NO: 25 is provided in the Sequence Listing.

10 Figure 7. DNA sequence which codes for dcAvd of SEQ ID NO: 2. For said DNA sequence also SEQ ID NO: 26 is provided in the Sequence Listing.

DETAILED DESCRIPTION OF THE INVENTION

There are two main reasons why there exist so many studies describing different chemical modifications and multiple genetically engineered mutants of avidin and streptavidin: the first motive is naturally connected to the colossal utilization of
 15 these proteins in a legion of applications. Therefore their physico-chemical properties and the biotin-binding properties have been regulated in aim to broaden the spectrum of applications and conditions wherein they can be used as well as to decrease some drawbacks that (strep)avidin-biotin system has. On the other hand,
 20 (strep)avidin-biotin pair offers for the scientist an interesting model system to study the structure and/or function related issues.

In the present application avidin-biotin complex was used as a model to radically engineer the topology of the multisubunit protein, whose N- and C-termini in distinct subunits are far away from each other, and do that so that the resultant
 25 avidin with four binding sites would be composed of two polypeptide chains. The task was accomplished by joining the two monomers that form the structural dimer in the wt avidin together. The resultant protein should maintain its structurally and functionally important properties as close as possible to the wt protein. Sanders et al. (Sanders, K.E., Lo, J., & Sligar, S.G., *Blood* 100, 299-305 (2002)) have succeeded
 30 to make such a maneuver when they first constructed circular permutant of the α -chain of hemoglobin and in the subsequent step they fused this novel subunit to the native α -unit. This fused pseudo-dimeric α -unit was able to oligomerize with the wt β -subunits and the resultant oligomer was functional in a way almost

indistinguishable from the wt oligomer. However this case was different when compared to the dual-chain avidin described in this application: only two subunits of the hetero-tetrameric complex were joined to single polypeptide chain and two other entered the complex as single units.

5 The natural topology of the wt avidin was modulated in order to arrange the termini of the subunits closer to each other. First two circularly permuted avidin monomers were constructed so that in the first mutant the termini were in the one end of the barrel whereas in the second mutant they situated in the opposite end. Because biotin binds to one end of the avidin barrel one could foresee that in the circularly
10 permuted avidin monomer, cpAvd6→5, where the new termini were introduced in that end it could affect the biotin binding. According to this assumption, in cpAvd6→5, where the new termini appeared to the loop between the β -strands 5 and 6 containing biotin-binding residues Trp70, Phe72 Ser73 and Ser75 (numbering according to wt avidin (Livnah, O. et al. *Proceedings of The National Academy of Sciences of the United States of America* 90, 5076-5080 (1993))), the biotin-binding
15 activity was reduced. One reason for the lowered affinity might be that the new free termini and therefore also these biotin-binding residues have more freedom to move compared to the corresponding loop in wt protein. When judged against previously mentioned circularly permuted streptavidin (Chu, V. et al. *Protein Sci* 7, 848-59 (1998)) the affinity of cpAvd6→5 was, however, preserved relatively well. Instead
20 in cpAvd5→4 where the new termini were introduced to the loop between the β -strands 4 and 5, which situates in the 3D-structure at the non-binding end of the barrel, no major changes were detected in its biotin-binding properties.

Interestingly, the dual-chain avidin (dcAvd) exhibited binding properties, which
25 were somewhere between these two circularly permuted forms. One apparent reason for that is the fact, that the dcAvd pseudo-tetramer has two considerably well preserved biotin-binding sites exhibiting strong affinity towards biotin originated from the cpAvd5→4 part and two biotin-binding sites with moderate affinity originated from the cpAvd6→5. On the other hand, in dcAvd two of the new
30 termini have been fused with the SGG spacer, which provides the transition from one monomer to the other. In this case it may rescue part of the structural stability of the wild-type loop (5→6) and therefore also restore part of the binding affinity.

The thermal stability and proteinase K durability of the circularly permuted avidin monomers and dual chain avidin was somewhat lower than that of the wild type
35 avidin. Proteinase K seemed to digest the new loops faster (not shown) than the

loop 3-4 that it is capable to break in the wt avidin (Ellison, D. et al. *Protein Sci* 4, 1337-45 (1995)). As cpAvd6→5 seemed to be more heavily glycosylated than cpAvd5→4 it may also explain why the molecular weight of the former mutant, according to HPLC, was slightly higher than that of the latter one.

- 5 Due to its structural symmetry the dual-chain avidin may result to two different quaternary structure assemblies. Depending on the outcome of this quaternary structure assembly the termini of both single-chain dimers may be orientated to the same or to the opposite directions in the pseudo-tetramer. From the applicative point of view the ideal position would be such that the functionally identical ends
10 would point to the same direction. Then it is possible to develop a system wherein, for example, the tighter binding sites bind to biotin containing surface, whereas the lower affinity binding sites point to solution. It is, however, possible to introduce non-symmetrical disulphide bridges between the single-chain dimers in order to fix their quaternary structure to the desired assembly.
- 15 While the preferred embodiment is described herein as derived from wild type avidin, it should be apparent to those skilled in the art that the circularly permuted avidin monomers and fusion proteins described herein can be made with variations of wild type avidin. Unless otherwise indicated or unless otherwise clear from the context, the term avidin is intended to encompass all forms of avidin including wild
20 type avidin, mutant forms of avidin, or variants of avidin, such as other poultry avidins comprising avidin proteins isolated from duck, goose, ostrich and turkey, and chicken avidin-related proteins (AVRs). The present invention could be applied even to other proteins, which bind biotin including bacterial streptavidin.

25 Circularly permuted proteins generally are created by disrupting the polypeptide chain at a selected point to create new termini and bridging the two natural termini either directly or through a linker such as an amino acid linker. Circular permutation thus has the effect of essentially preserving the sequence and identity of the amino acids of a protein while generating new termini at different locations. Moreover, the tertiary structure of the protein is generally conserved.

- 30 There are two general requirements for the creation of a circularly permuted protein that retains its native biological activity: 1) the termini in the native protein must be favorably located so that creation of a linkage does not destroy biological activity; and 2) there must exist an "opening site" where new termini can be formed without disrupting a region critical for protein folding and desired biological activity. In

some cases it is possible to modify biological activity, such as biotin binding in the case of avidin.

Generally, linkers are molecules that contain two reactive sites, one that will form a covalent bond with the carboxyl terminal amino acid and one that will form a covalent bond with the amino terminal amino acid. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. The most common and simple example is a peptide linker that consists of several amino acids joined through the peptide bonds to the termini of the native protein. The linkers may be joined to the terminal amino acids through their side groups. However, in a preferred embodiment, the linkers will be joined through peptide bonds to the alpha carbon amino and carboxyl groups of the terminal amino acids. Generally, neutral amino acids and/or amino acids with small side chains are preferred, such as glycine, alanine, and serine.

It is preferable to use a linker that preserves the spacing between the termini comparable to the unpermuted or native molecule, particularly if the desire is to maintain or improve the native biological activity of the molecule. For circularly permuted avidin, the linker is composed of several amino acids, preferably about one to six amino acids. Most preferably, the linker is a peptide of four glycine residues and two serine residue, in the order GGSGGS (SEQ ID NO:3). The first glycine is attached to the carboxyl terminal amino acid of the native protein and the serine is attached to the amino terminal amino acid of the native protein.

The selection of an opening site may be determined by a number of factors. One factor is whether the biological function is to be preserved or altered. If the biological function is not to be altered, the opening site should be away from the active site and away from other structurally or functionally important sites. For example, preferred opening sites will be located in regions that do not show a highly regular three-dimensional structure such as alpha helices, pleated sheets, β -barrel structures, and the like. However, if the object is to alter the biological function, it may be more advantageous to open the molecule near the active site. For example, to lower the biotin binding affinity of avidin, the opening site can be located on the flexible loop near the biotin binding site that contains amino acids that participate in biotin binding. In the present invention the opening site was situated between the β -sheets 4 and 5 in the first monomer and between the β -sheets 5 and 6 in the second monomer.

The fusion protein described herein includes two avidin monomers, which have been circularly permuted (cpAvd5→4 and cpAvd6→5). The two proteins may be fused together directly or joined by means of a spacer, such as a peptide spacer. The peptide spacer may range from about 1 to 40 residues in length, but also other
5 spacers with alternative length or composition may be used. It is desirable to retain full or partial biological activity of both monomers. The length and characteristics of the spacer will be important in achieving this objective. Generally the spacer connecting the two monomers has no biological activity itself and functions only to link and provide some distance between the two active proteins forming the fusion
10 protein. However, one of skill will recognize that the residues of the spacer may be chosen to optimise a property of the fusion protein. Here preferably a spacer of three amino acids, one serine and two glycine residues, is used (SGG).

The circularly permuted avidin monomers (cpAvd5→4 and cpAvd6→5) could be further mutagenized by site specific mutagenesis before fusion in order to construct
15 dual-chain avidin monomers, which bear, for example, two different kinds of binding sites. Hence, the binding affinity or specificity in the two neighbouring binding sites in dual-chain avidin monomer can be different from each other. In the present invention the cpAvd5→4 and/or cpAvd6→5 derived parts of dcAvd were mutated. The monomer can be mutated by changing the tyrosine residue 33 to any
20 other amino acid residue X and/or isoleucine residue 117 to any other amino acid residue X, (Y33X, I117X). Preferably the monomer can be mutated by changing the tyrosine residue 33 to histidine residue and/or the isoleucine residue 17 to cysteine residue, (Y33H, I117X). Owing to these modifications dcAvd mutant, p54(I117C)+p65(Y33H), contains two high-affinity binding sites and two low-
25 affinity binding sites.

The circularly permuted proteins and fusion proteins may be made using methods known to those of skilled in the art. These include chemical synthesis, modifications of existing proteins, and expression of circularly permuted proteins using recombinant DNA methodology. The fusion protein can be made as a single
30 polypeptide or the second peptide can be attached to the base polypeptide after separate synthesis of the two component polypeptides. The circularly permuted protein and/or fusion protein may be made by chemically modifying a native or preexisting protein. Generally, this requires reacting the native protein in the presence of the linker to form covalent bonds between the linker and the carboxyl
35 and amino termini of the protein, thus forming a circular protein. New termini are

then formed by opening the peptide bond joining amino acids at another location. This may be accomplished chemically or enzymatically using, for example, a peptidase.

5 In the preferred embodiment, the circularly permuted proteins, and/or fusion proteins including the circularly permuted proteins, will be synthesized using recombinant methodology. Generally, this involves creating a polynucleotide sequence that encodes the circularly permuted base polypeptide (or the entire fusion protein containing the base polypeptide), placing the polynucleotide in an expression cassette under the control of a suitable expression promoter, expressing
10 the protein in a host, isolating the expressed protein and, if required, renaturing the protein. If the secondary protein is made separately it is then ligated to the circular permutant.

DNA encoding a circularly permuted polypeptide or fusion protein including the circularly permuted polypeptide can be prepared by any suitable method, including,
15 for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang et al. Meth. Enzymol. 68: 90-99 (1979); the phosphodiester method of Brown et al., Meth. Enzymol. 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage et al., Tetra. Lett., 22: 1859-1862 (1981); and the solid support method of U.S. Pat.
20 No. 4,458,066. Alternatively, partial length sequences may be cloned and the appropriate partial length sequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence. In a preferred embodiment, DNA encoding the circularly permuted polypeptide will be produced using DNA amplification methods, for example polymerase chain
25 reaction (PCR).

The circularly permuted proteins and their fusion proteins may be expressed in a variety of host cells, including E coli, other bacterial hosts, yeast, and various higher eukaryotic cells, such as the COS, CHO and HeLa cells lines, insect cells, and myeloma cell lines, as well as in different plant production systems (corn, tobacco etc.) and in transgenic animals (rodent, bovine etc.). Even production in
30 vitro could be profitable. In a preferred embodiment of the present invention the fusion protein is encoded by a plasmid or a viral vector. The recombinant protein gene is operable linked to appropriate expression control sequences for each host.

The DNA constructs encoding the circularly permuted proteins or fusion protein can be transferred to be expressed into the chosen host system by methods well-known for those skilled in the art.

5 Once expressed, the recombinant base circularly permuted protein or fusion protein can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity chromatography, gel electrophoresis and the like. Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the
10 polypeptides may then be used as desired.

After chemical synthesis, biological expression, or purification, the circularly permuted polypeptide and/or fusion protein may possess a conformation substantially different than the native protein. In this case, it may be necessary to denature and reduce the protein and then to cause the protein to re-fold into the
15 preferred conformation.

To determine which circularly permuted polypeptides or fusion proteins are preferred, the proteins should be assayed for biological activity. Such assays, well known to those of skilled in the art, generally fall into two categories; those that measure the binding affinity of the protein to a particular target, and those that
20 measure the biological activity of the protein. Also structural evaluations are made.

The fusion proteins of the present invention are useful for a variety of applications such as separations, drug delivery, targeting, and in diagnostic assays. For example, an avidin fusion protein can be bound to a biotinylated substrate. The biological activity of the secondary molecule can then be used, for example, to capture and
25 separate a particular molecule out of an impure solution. The purified molecule is then dissociated from the fusion protein. The lower biotin affinity of the fusion protein as compared to that of wild type avidin allows release of the fusion protein from the substrate and reuse of the fusion protein. The two binding pockets in the dcAvd may be of different kind but are the same two in each dcAvd. The dual-chain
30 pseudo-tetrameric avidin containing two of these dcAvids has thus two plus two of these binding pockets. The two strong specificity of the fusion protein can be used similarly in other applications.

Besides of biotin, dcAvidin could be engineered to bind some different ligands with half of the binding sites. Furthermore this dual-ligand protein platform could be modified by the means of site directed mutagenesis, molecular pharming or random mutagenesis to bind two different ligands regardless of biotin.

- 5 Dual chain avidin (dcAvd) can also be used as a fusion-partner. Due to the design of the molecule one fusion protein, dcAvd, contains two binding sites (in contrast to only one as would be the case with wild-type avidin) and upon “pseudo-tetramer” formation the functional quaternary structure can be attained as a result of dimerization of the dcAvd and not tetramerization as would be the case with wild-
10 type avidin.

The constructed dual chain avidin (dcAvd) provides a structural scaffold for avidins with mixed affinity properties. Therefore its variants have enormous value in applications. This rationale derives from the fact that the dual-chain avidin is a genetically fused entity, which enables the engineering of the avidin subunit
15 properties separately. It is possible to maintain the high affinity towards biotin by two of the binding sites while modifying the affinity in other two sites as desired. One preferred embodiment is to change two of the binding sites reversible which enables mild detachment of bound materials by free biotin (Laitinen,O.H. et al. *FEBS Lett* 461, 52-8 (1999), Laitinen,O.H. et al. *Biochem J* 363, 609-17 (2002),
20 Qureshi,M. & Wong,S. *Protein Expr. Purif.* 25, 409 (2002), Qureshi,M.H. et al. *J Biol Chem* 2, 2 (2001)). Still another preferred embodiment is to modify half of the binding sites so that it is possible to attach to their close proximity such smart polymer conjugates that are able to respond to changes in pH, light intensity and temperature (Ding,Z. et al. *Nature* 411, 59-62 (2001), Shimoboji,T. et al. *Bioconjug*
25 *Chem* 12, 314-9 (2001)) and thereafter alter the binding characteristic of the modified sites by adjusting these physical/chemical parameters.

In single-chain avidin (scAvd), where two dcAvd-molecules are fused together via a linker to form a single polypeptide with four binding sites for biotin, each of the four binding sites can be modified by protein engineering independently. Therefore,
30 it is possible to have even four different binding sites in one polypeptide, all of which are also located identically in the quaternary structures of these proteins. This allows construction of divergent protein tools, in which ligand binding properties, ligand specificities and/or, for example, loop displayed peptides and other modifications can be positioned exactly as desired.

As a fusion partner scAvd provides completely new possibilities, because the four binding sites in one polypeptide constitute the whole quaternary structure, there is no need for scAvd containing fusion protein to form oligomeric assemblies, providing that the other fusion partner is

- 5 also monovalent. Owing to this property, scAvd can be fused easily to multivalent proteins and even membrane proteins like receptors.

The present invention is further described by the following non-limiting example.

EXAMPLE 1

Planning of the constructs

- 10 The 3D structure of avidin tetramer was examined in aim to find those β -strands of the barrels where the polypeptide transition from one subunit to the other would create as little steric disturbance as possible. The study revealed that in the quaternary structure, β -strand 4 in one subunit and strand 6 in another subunit of the structural dimer are juxtaposed. Moreover, when β -strand 4 goes up in one barrel,
- 15 the β -strand 6 goes down in the neighbouring subunit. Therefore the loops 4 \rightarrow 5 and 5 \rightarrow 6 of the neighbouring subunits were used as a monomer-monomer transition point in the planned fused dual-chain avidin, dcAvd (SEQ ID NO:2), (Fig.1A and B). The determination of the transition point consequently predestined the places of the termini of the circularly permuted avidins to be as following: N-
- 20 terminus of the first circularly permuted avidin monomer (cpAvd5 \rightarrow 4) is located before the β -strand 5 in the wild-type sequence and the new C-terminus followed the β -strand 4. Correspondingly, the second circularly permuted avidin monomer (cpAvd6 \rightarrow 5) started just before the β -strand 6 and ended after the β -strand 5.

- 25 In the circularly permuted avidin monomers, cpAvd5 \rightarrow 4 and cpAvd6 \rightarrow 5; the original N- and C- termini were fused with an artificial linker composed of a hexapeptide GGSGGS (SEQ ID NO:3). Both constructs started with the normal signal sequence of avidin followed by the three first N-terminal amino acid residues of native avidin to ensure the correct cleavage by signal peptidase. However, other signal sequences could be used and the three first N-terminal amino acid residues of
- 30 native avidin could be left out. Finally, the dual-chain avidin was produced by fusing together the two circularly permuted avidin monomers described above (Fig. 1A and B). A tri peptide spacer SGG was used to connect the C terminus of

cpAvd5→4 to the N terminus of cpAvd6→5 and the resultant dual chain fusion was designated as dcAvd.

DNA constructs and recombinant baculoviruses

In the first step in generation of the circularly permuted avidin monomers the signal sequence of avidin plus the two first amino acids of avidin were PCR amplified from avidin cDNA with forward primer AK33 (5'-CTGCTAGATCTATGGTGCACGCAACCTCCCC-3') (SEQ ID NO:4)

and reverse primer Sig-2 (5'- cctggc agagaggccggga -3') (SEQ ID NO:5) the product was digested with BglII and ligated into BamHI and StuI treated pFASTBAC1 plasmid (Bac-To-Bac™, Gibco BRL, Life Technologies, Gaithersburg, MD, USA).

In the next step in the case of cpAvd5→4 the region between beta strands 5 and 8 was PCR amplified with forward primer 54N1 5'- aag agg acc cag ccc acc tt -3' (SEQ ID NO:6) (coding also for the third amino acid, K, of avidin) and reverse primer 54C1 5'- gga gcc tcc gga gcc tcc ctc ctt ctg tgt gcg cag -3' (SEQ ID NO:7) (which inserts also the GGSGGS linker after the beta strand eight). At the same time in a different tube the region between the beta strands 1 and 4 was PCR amplified with forward primer 54N2 5'- gga ggc tcc gga ggc tcc gcc aga aag tgc tgc ctg -3' (SEQ ID NO:8) (which codes also for the GGSGGS linker, and the sequence is identical with the GGSGGS coding part present in 54C1) and reverse primer 54C2 5'- tgggc aagct tca ctt gtt gat ggt gtt ttg-3' (SEQ ID NO:9) (which contains a stop codon and a HindIII restriction site). These two PCR products were purified and used as a template in the subsequent PCR step, in which the fragments containing complementary regions (the GGSGGS region) were combined and amplified with the terminal primers (54N1 and 54C2). This product was treated with HindIII and ligated into StuI and HindIII treated pFASTBAC1 derivative containing the signal sequence and the first two amino acids.

Similarly in the case of cpAvd6→5 the region between beta strands 6 and 8 was PCR amplified with forward primer 65N1 5'- aag tcc acc act gtc ttc acg -3' (SEQ ID NO:10) (which adds also the third amino acid, K, of avidin) and reverse primer 54C1, as previously described, (which inserts also the GGSGGS linker after the beta strand eight). In a different tube the region between beta strands 1 and 5 was PCR amplified with forward primer 54N2, as previously described, (which codes

- also for the GGSGGS linker, and the sequence is identical with the GGSGGS coding part present in 54C1) and reverse primer 65C2 5'- agaca aagct tca ctc tga aaa ctt cca att g -3' (SEQ ID NO:11) (which contains a stop codon and a HindIII restriction site). These two PCR products were purified and used as a template in the subsequent PCR step, in which the fragments containing complementary regions (the GGSGGS region) were combined and PCR amplified with the terminal primers (65N1 and 65C2). This product was treated with HindIII and ligated into StuI and HindIII treated pFASTBAC1 derivative containing the signal sequence and the first two amino acids.
- 10 The dcAvd was constructed using the two above described constructs (cpAvd5→4 and cpAvd6→5) as templates. In the first step cpAvd5→4 was PCR amplified with forward primer AK33, described above, and reverse primer dual1 5'-gtggtgatccgccgactgttgatggtgtttgtgt-3' (SEQ ID NO:12) (which codes also for the SGG monomer to monomer linker and contains a BamHI restriction site. This product was digested with BglII and BamHI and ligated into BamHI treated pFASTBAC1 and named pFASTBAC1(cpAvd5→4(no-stop)). In the next step cpAvd6→5 was PCR amplified with forward primer dual2 5'-ccggcgatccaccactgtcttcacgggc-3' (SEQ ID NO:13) (which also codes for the SGG linker and contains a BamHI restriction site) and reverse primer 65C2, as described above, (which contains a stop codon and a HindIII restriction site). This product was digested with BamHI and HindIII and ligated into previously obtained pFASTBAC1 derivative containing cpAvd5→4 and the spacer SGG, which was also treated with BamHI and HindIII.

EXAMPLE 2

25 Production and purification of the mutant avidins

- The circularly permuted avidin monomers and the dual-chain avidin were produced in baculovirus infected insect cells (Bac-To-Bac™, Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and they were purified from the cell extracts by 2-aminobiotin-agarose affinity chromatography essentially as described in detail elsewhere (Laitinen, O.H. et al. *FEBS Lett* 461, 52-8 (1999), Airenne, K.J. et al. *Protein Expression and Purification* 9, 100-108 (1997)). In the baculovirus expression system the pFASBAC1 plasmid serves as a donor vector and the expression cassette, including the coding region for cpAvd5→4, cpAvd6→5 and dcAvd can be transferred from it using site specific transposition into recombinant

baculovirus genomes (according to the manufacturers instructions). These recombinant genomes were used in transfections of the insect cells, in which the primary recombinant baculovirus stocks were generated. These stocks were used in consequent insect cell infections.

- 5 Briefly, the insect cell infections were carried out with cells that were transferred to a biotin-free medium. Infections were allowed to proceed for 72 hours, and after that the cells were collected by centrifugation (1500 g, 5 min). The cell pellet was dissolved into a lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, 1 % Triton X-100, pH 8) and incubated on ice for 30 minutes. After that the cell extract
10 was sonicated and centrifuged (15000 g 20 min) and the pH of the supernatant was adjusted to 11 with 5 M NaOH and NaCl was added to a final concentration of 1 M. Then the cell extract was applied to a 2-iminobiotin agarose column washed previously with binding buffer (50 mM Na-carbonate, 1 M NaCl). Bound proteins were eluted from the column with 50 mM Na-acetate, pH 4.

15 EXAMPLE 3

Biotin binding experiments

- Reversibility of biotin binding and affinity towards 2-iminobiotin were determined with an IAsys optical biosensor (Affinity Sensors, Cambridge, UK) (Table 1.) as described in detail by Marttila et. al. (Marttila, A.T. et al. *FEBS Lett* 441, 313-7
20 (1998)). Briefly, The 2-iminobiotin ligand was immobilized onto the carboxymethyldextran surface of a cuvette using N-hydroxysuccinimide activation. Binding of various concentrations of avidin or avidin mutants onto 2-
iminobiotinylated surface was measured in a 50 mM borate buffer (pH 9.5) containing 1 M NaCl at 20 °C. The 2-iminobiotin cuvettes were regenerated with 20
25 mM HCl. The kinetic rate constants for association (k_{ass}) and dissociation (k_{diss}) were calculated using the Fast Fit program package (Affinity Sensors). The obtained rate constants were used to calculate $K_d(\text{rel})$ according to $K_d = k_{\text{diss}}/k_{\text{ass}}$. Equilibrium data obtained from measurements with different protein concentrations were used to calculate the dissociation constant $K_d(\text{eq})$.
- 30 Table 1. The association (k_{ass}) and dissociation (k_{diss}) rate constants and the affinity towards 2-iminobiotin for different avidins were determined with an IAsys optical biosensor. $K_d(\text{eq})$ was determined experimentally from the binding curves whereas

the $K_d(\text{rel})$ was calculated from the rate constants. The reversibility value corresponds to reversibility from biotin surface.

Protein	$k_{\text{ass}} (\text{M}^{-1}\text{s}^{-1})$	$k_{\text{diss}} (\text{s}^{-1})$	$K_d(\text{eq}) (\text{M})$	$K_d(\text{rel}) (\text{M})$	reversibility
wt Avd	$(5.5 \pm 0.5) \times 10^5$	$(1.9 \pm 1.4) \times 10^{-3}$	$(2.2 \pm 1.0) \times 10^{-8}$	$(3.4 \pm 2.5) \times 10^{-8}$	9 %
cpAvd5→4	$(2.6 \pm 0.3) \times 10^5$	$(1.6 \pm 0.7) \times 10^{-3}$	$(1.5 \pm 0.5) \times 10^{-8}$	$(5.9 \pm 2.6) \times 10^{-8}$	9 %
cpAvd6→5	$(3.9 \pm 1.2) \times 10^4$	$(3.9 \pm 2.2) \times 10^{-3}$	$(5.5 \pm 2.3) \times 10^{-7}$	$(9.9 \pm 7.6) \times 10^{-7}$	16 %
dcAvd	$(9.8 \pm 1.3) \times 10^4$	$(1.1 \pm 0.5) \times 10^{-3}$	$(4.1 \pm 1.2) \times 10^{-8}$	$(1.1 \pm 0.5) \times 10^{-7}$	8 %

- 5 The reversibility of biotin binding was also measured using IAsys apparatus. In the reversibility assay, the sample proteins were allowed to bind to the biotinylated cuvette surface in PBS containing 1 M NaCl. After the equilibrium was reached, the cuvette was filled with PBS, 1 M NaCl containing biotin (423 $\mu\text{g} / \text{ml}$) and the dissociation of the proteins was monitored for one hour.
- 10 The colorimetric HABA-assay was performed essentially as described by Green (Green, N. *Methods in Enzymology* 18, 418-424 (1970)). Dual-chain avidin (dcAvd) in 100 mM phosphate buffer (pH 7) was saturated with excess HABA and the absorbance was measured at 500 nm with a Beckman DU640 spectrophotometer. After that, excess biotin, which replaces HABA from the binding site, was added to
- 15 the sample and the absorbance at 500 nm was measured again. The observed change in the absorbance was used to calculate the number of free binding sites in dcAvd.

CpAvd5→4 showed similar biotin-binding characteristics as the wt avidin in both assays whereas the circularly permuted monomer cpAvd6→5 was more reversible

and it exhibited reduced affinity as compared to the wt avidin. Interestingly, the dual chain avidin dcAvd showed similar reversibility as wt avidin and its affinity to 2-iminobiotin was decreased only negligibly. The number of biotin-binding sites per dual-chain avidin pseudo-tetramer (is actually a dimer with four binding sites) was determined in a colorimetric HABA-assay according to Green (Green, N. *Methods in Enzymology* 18, 418-424 (1970)). The average from two independent experiments gave an approximation of 3.3 free biotin-binding sites per dimeric molecule (pseudo-tetramer).

EXAMPLE 4

10 Structure analyses

Quaternary status of the avidin mutants were determined by high performance liquid chromatography (HPLC), using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech AB) connected to a Shimadzu HPLC system with SCL - 10A VP system controller, RF-10A XL fluorescence detector and SPD-
15 M10A VP diode array detector. The data obtained was processed with Class VP 5.03 program. As a running buffer we used 50 mM Na-phosphate, 650 mM NaCl, pH 7.2. All runs were performed with flow speed of 0.5 ml / min and the molecular weight markers were BSA (68 kDa), ovalbumin (43 kDa) and cytochrome c (12.4 kDa).

20 According to gel filtration chromatography performed in the absence of biotin the deduced molecular weights (Table 2) indicated that all constructs had folded correctly and formed quaternary structures like that of the wt avidin; two dual-chain avidins formed a pseudo-tetramer. In the denaturing SDS-PAGE both circularly permuted avidin monomers had apparent molecular weights corresponding to a wt
25 monomeric form (Fig. 2), whereas dcAvd had a molecular weight of ≈ 32 kDa which corresponds well to its theoretical molecular weight. Moreover, we observed also that cpAvd6 \rightarrow 5 was more heavily glycosylated than cpAvd5 \rightarrow 4.

EXAMPLE 5

Stability analyses

30 Thermal stability of the mutants was studied by using a SDS-PAGE-based method essentially as described in detail by Bayer et al. (Bayer, E.A. et al. *Electrophoresis* 17, 1319-1324 (1996)). Briefly, the protein sample in the absence or presence of

biotin was diluted to a final concentration of 0.2 mg/ml in 50 mM Na-carbonate buffer, and the proteins were acetylated by adding acetyl NHS ester. Equal amount of SDS-PAGE sample buffer was added to each sample and the protein was incubated at different temperatures (between 25-100 °C) for 20 minutes. After that the samples were subjected to SDS-PAGE analysis and the gel was finally stained with Coomassie brilliant blue. Dissociation of the proteins into their subunits was observed from the gels and the transition temperatures, wherein half of the protein was (pseudo-)tetrameric and half monomeric were determined.

Proteinase K assay was performed essentially as described by Laitinen et al. (Laitinen, O.H. et al. *FEBS Lett* 461, 52-8 (1999)). Briefly, protein samples (50 µl, 1 mg/ml) with or without three times molar excess of biotin were diluted with equal volume of 100 mM Tris-HCl, pH 7.8 and incubated in the presence of proteinase K (1:25 w/w) at 37 °C. Samples were taken at designated time intervals and stored at –20 °C. SDS-PAGE sample buffer was added to each sample before they were boiled and subjected to SDS-PAGE. The amount of intact avidin monomer present in the samples was determined from Coomassie brilliant blue stained gels.

The results are shown in Table 2 and revealed that the mutants were not quite as stable as the wt avidin. The durability of the mutants in the presence of proteinase K was also tested (Fig. 3) and all constructs were more prone for cleavage than wt avidin.

Table 2. The molecular weights (MW) were deduced from the gel filtration experiments. The stability parameters were obtained from heat/SDS-PAGE analysis. Transition temperature (T_r) is a temperature wherein half of the tetramer/pseudo-tetramer was broken into monomers in the presence (+btn) or absence of biotin.

Protein	MW (kDa)	T_r (°C)	T_r +btn (°C)
wt Avd	62	60	95
cpAvd5→4	52	45	75
cpAvd6→5	56	40	70
dcAvd	56	40	75

EXAMPLE 6.

Production of a mutated dual-chain avidin (dcAvd).

Mutagenesis of cpAvd5→4 and cpAvd6→5.

- 5 The numbering of the mutagenised amino acid residues in cpAvd molecules (Y33H, I117C) is according to mature chicken avidin amino acid residue numbering (SEQ ID NO:1. The signal peptide is not included in numbered amino acid residues. 24 first amino acid residues of the sequence belong to signal sequence and are processed away during the posttranslational modification of the avidin).
- 10 pFASTBAC1(cpAvd5→4(no-stop))-plasmid was mutagenised by QuikChange method using oligonucleotides Q1 (5'-AGG GTC GGC TCG AAC ATC TT) (SEQ ID NO:14) and Q2 (5'-AAG ATG TTG CAG CCG ACC CT) (SEQ ID NO:15). The resulting plasmid was sequenced and named pFASTBAC1 (p54(I117C)).
- 15 pFASTBAC1-plasmid containing cDNA of cpAvd6→5 was mutagenised by QuikChange method using oligonucleotides Y33H.1 (5'-CAC AGG CAC CCA CAT CAC AGC CG) (SEQ ID NO:16) and Y33H.2 (5'-CGG CTG TGA TGT GGG TGC CTG TG) (SEQ ID NO:17). The resulting plasmid was sequenced and named pFASTBAC1 (p65(Y33H)).

EXAMPLE 7.

- 20 Cloning of pFASTBAC1(p54(I117C)+p65(Y33H)).

The pFASTBAC1(p65(Y33H)) was used as a template in PCR-reaction with primers dual2 and 65C2. The resulting product was extracted from 1% agarose gel, digested with BamHI/HindIII and ligated to pFASTBAC1 (p54(I117C)) digested with BamHI and HindIII. The resulting plasmid was sequenced.

- 25 Expression of p54(I117C)+p65(Y33H) was carried using Bac-to-Bac expression system. 2-iminobiotin affinity chromatography was used to isolate the protein from infected insect cells.

EXAMPLE 8.

Dissociation analysis of p54(I117C)+p65(Y33H).

The biotin-binding properties of produced mutagenised dcAvds were analysed by method based on the quenching of biotin-coupled fluorescent probe ArcDia BF560 (ArcDia Ltd., Turku, Finland) due to binding to avidin (Figure 4). PerkinElmer LS55 luminometer was used for measurements. Figure 4 shows the quenching of biotin-coupled BF560 by avidin. The spectra of 50 nM biotin-Bf560 solution in 50 mM Na-phosphate containing 650 mM NaCl was measured (red). Chicken avidin (Belovo S. A., Bastogne, Belgium) was added to final subunit concentration of 83 nM and green spectra was obtained.

Briefly, 50 nM free biotin-Bf560 solution in 50 mM Na-phosphate containing 650 mM NaCl was measured using excitation at 560 nm (2.5 nm slit) and emission was collected at 578 nm (5 nm slit). Continuous stirring (setting low) was used throughout the analysis. After 100 sec measurement, the protein under study was added to final biotin-binding subunit concentration 50 nM and the measurement was continued for 500 sec. After that, free biotin was added to final concentration of 5 μ M and the signal was measured for 3600 sec at room temperature (23 ± 0.5 °C) (Figure 5). Figure 5 shows the dissociation of biotin-coupled fluorescent probe (ArcDia BF560) with excess unlabeled biotin from proteins analyzed in this example. Avd(Y33H) (Marttila, A. T. et al. *Biochem J.* 369, 249-254 (2003)) shows rapid and complete dissociation of the fluorescent biotin probe from the four identically mutagenised (Y33H) subunits of tetramer (black). p54(I117C)+p65, instead, showed slow dissociation of the fluorescent biotin probe (green), comparable to that of wild-type avidin (not shown). Only half of the binding sites of p54(I117C)+p65(Y33H) have the mutation, and approximately half of the bound biotinylated probe is released within one hour (red).

EXAMPLE 9.

Construction of expression vector of single-chain avidin (scAvd).

In this example two dcAvd-molecules are fused together tail-to-head via 12 amino-acid linker (GGSGSGSGSGSG) to form a polypeptide with four binding sites for biotin. Other forms of linker may be also used.

The pFASTBAC1-plasmid containing dual-chain avidin (dcAvd) sequence was mutagenized using QuikChange method with primers poisto1 (5'-GGC GGA TCT ACC ACT GTC) (SEQ ID NO:18) and poisto2 (5'-GAC AGT GGT AGA TCC GCC) (SEQ ID NO:19) in order to destroy the BamHI restriction site in the

sequence. The obtained plasmid was sequenced and named pFASTBAC1 (p54+p65-poisto).

pFASTBAC1 (cp6→5) was used as a template in PCR reaction with primers Single2.2 (5'- CCG GCA GAT CTA CCA CTG TCT TCA CGG GC) (SEQ ID NO:20) and Malooppi65.4 (5'-ATC CTC GGA TCC CGA TCC GGA ACC TCC CTC TGA AAA CTT C) (SEQ ID NO:21). The primer Single2.2 includes a BglII site. The primer Malooppi65.4 extends the sequence of cpAvd6→5 at the C-terminus with sequence GSGSGS and includes a BamHI site. The obtained PCR-product was extracted from 1% agarose gel, digested with BamHI and BglII and cloned to BamHI-digested pFASTBAC1(cpAvd5→4(no-stop))-plasmid. The obtained plasmid was sequenced and named pFASTBAC1(p54+p65looppi).

pFASTBAC1 (p54+p65-poisto) was used as a template in PCR reaction with primers MALooppi54 (5'- GGC TCT GGT GGC TGG ATC CGG CTC TGG CAG CGG CAG GAC CCA GCC C) (SEQ ID NO:22) and A414 (5'- CTA CAA ATG TGG TAT GGC TG) (SEQ ID NO:23). The primer Malooppi54 extends the sequence of cpAvd5→4 at the N-terminus with sequence GSGSGSG and includes a BamHI site. The obtained PCR-product was extracted from 1% agarose gel and cloned to pGEM-T-easy vector using TA-cloning method. The obtained plasmid was sequenced and named pGemTeasy(p54looppi+p65).

The pGemTeasy(p54looppi+p65) was digested with BamHI/HindIII, the obtained DNA-fragment was extracted from 1% agarose gel and cloned to BamHI/HindIII-digested pFASTBAC1(p54+p65looppi). The resulting plasmid was named pFASTBAC1(scAvd).

Expression of singlechain-avidin (SEQ IDNO:24) will be performed according to instructions of Bac-to-Bac manual.

It is understood that the disclosed invention is not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

Claims

1. A circularly permuted avidin monomer, **characterized** in that the carboxyl terminal amino acid and the amino terminal amino acid of the polypeptide of the avidin monomer have been joined directly or via a linker, and new carboxyl and amino termini have been created by cleavage of the polypeptide and the resulting circularly permuted avidin monomer binds biotin.
2. The circularly permuted avidin monomer of claim 1, **characterized** in that the avidin is selected from wild type avidin, mutant form of avidin, or variant of avidin, such as other poultry avidins and chicken avidin-related proteins (AVRs).
3. The circularly permuted avidin monomer of claim 1, **characterized** in that the carboxyl terminal amino acid and amino terminal amino acid have been joined by a linker comprising one or more amino acids.
4. The circularly permuted avidin monomer of claim 3, **characterized** in that the linker is a hexapeptide comprising four glycine residues and two serine residues and wherein one glycine is connected to the carboxyl terminal amino acid and one serine is connected to the amino terminal amino acid.
5. The circularly permuted avidin monomer of claim 1, **characterized** in that the circularly permuted monomer is cpAvd5→4.
6. The circularly permuted avidin monomer of claim 1, **characterized** in that the circularly permuted monomer is cpAvd6→5.
7. The circularly permuted avidin monomer of claim 5 or 6, **characterized** in that the monomer has been mutated.
8. The circularly permuted avidin monomer of claim 5 or 6, **characterized** in that the monomer has been mutated by changing the tyrosine residue 33 to any other amino acid residue X and/or the isoleucine residue 117 to any other amino acid residue X, (Y33X, I117X).
9. The circularly permuted avidin monomer of claim 5 or 6, **characterized** in that the monomer has been mutated by changing the tyrosine residue 33 to histidine residue and/or the isoleucine residue 117 to cysteine residue, (Y33H, I117C).

10. A dual-chain avidin (dcAvd), **characterized** in that it comprises a fusion of the circularly permuted monomer cpAvd5→4 and the circularly permuted monomer cpAvd6→5, or the mutated monomer forms of claim 7, 8, or 9.
- 5 11. A dual-chain avidin of claim 10, **characterized** in that the circularly permuted monomer cpAvd5→4 and the circularly permuted monomer cpAvd6→5, or the mutated monomer forms of claim 7, 8, or 9 are fused together directly or joined by means of a spacer.
12. A dual-chain avidin of claim 11, **characterized** in that the spacer is a peptide spacer from about 1 to 40 amino acid residues.
- 10 13. A dual-chain avidin of claim 12, **characterized** in that the spacer is a peptide SGG.
14. The circularly permuted avidin monomer of claim 1, **characterized** in that the biotin-binding affinity of the circularly permuted avidin is different from the wild type avidin biotin-binding affinity.
- 15 15. A dual-chain pseudo-tetrameric avidin, **characterized** in that it comprises two dual-chain avidins (dcAvd).
16. A single-chain avidin (scAvd), **characterized** in that it comprises two dual-chain avidin (dcAvd) molecules of claim 10 fused together to form a single polypeptide.
- 20 17. A single-chain avidin of claim 16, **characterized** in that the dcAvd-molecules are fused together via a linker.
18. A single-chain avidin of claim 17, **characterized** in that the linker is a 12 amino-acid linker GGSGSGSGSGSG.
19. An isolated polynucleotide encoding any of the avidin proteins of claims 1-18.
- 25 20. A recombinant vector comprising the polynucleotide of claim 19, wherein the polynucleotide is DNA.
21. A recombinant host cell comprising the polynucleotide of claim 20, wherein said polynucleotide is DNA.

22. A method for producing a polypeptide comprising expressing from the recombinant cell of claim 21 the polypeptide encoded by said polynucleotide.

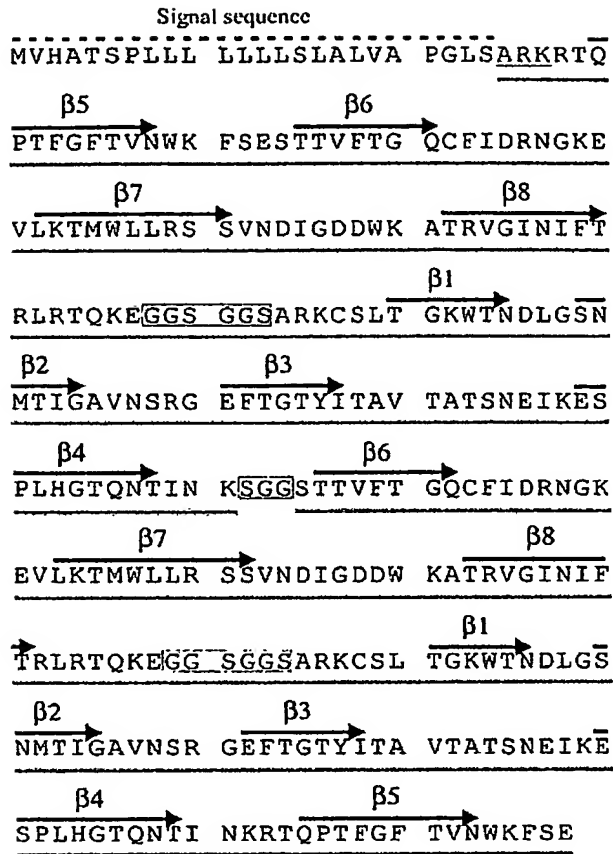
(57) Abstract

Two circularly permuted avidin monomers are designed. The circularly permuted monomers (cpAvd5→4 and cpAvd6→5) are fused and the resulting fusion peptides (dcAvd) form a pseudo-tetrameric dual-chain avidin, which is biologically active in biotin binding and shows similar structural characteristics as wild-type avidin. The dcAvd makes the development of dual-affinity avidins possible by allowing the adjustment of the ligand binding properties in the half of the binding sites differently than in the rest of the sites. The present invention provides further a single-chain avidin (scAvd) where two dcAvd-molecules are fused together via a linker to form a single polypeptide with four binding sites for biotin.

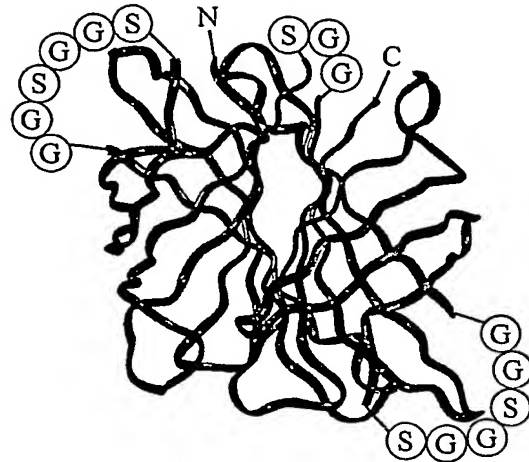
Fig. 1

Fig. 1

A



B



L 5

Fig. 2

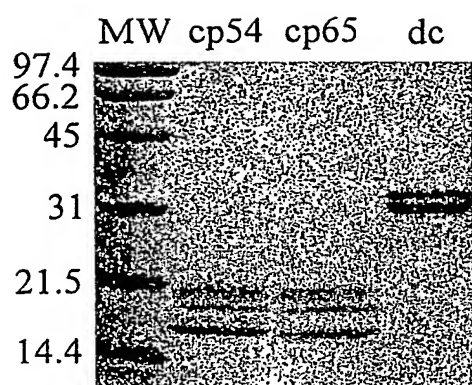


Fig. 3

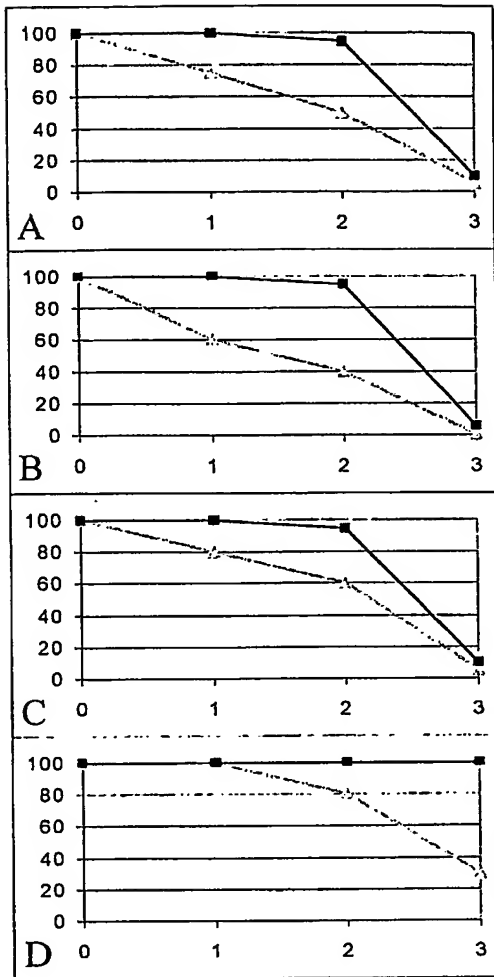
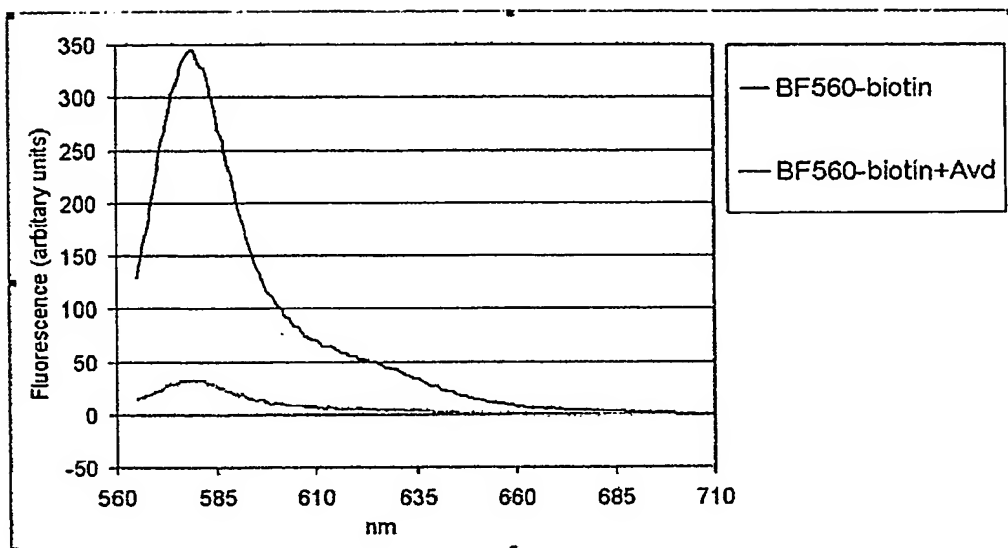


Fig. 4



L5

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Fig. 5

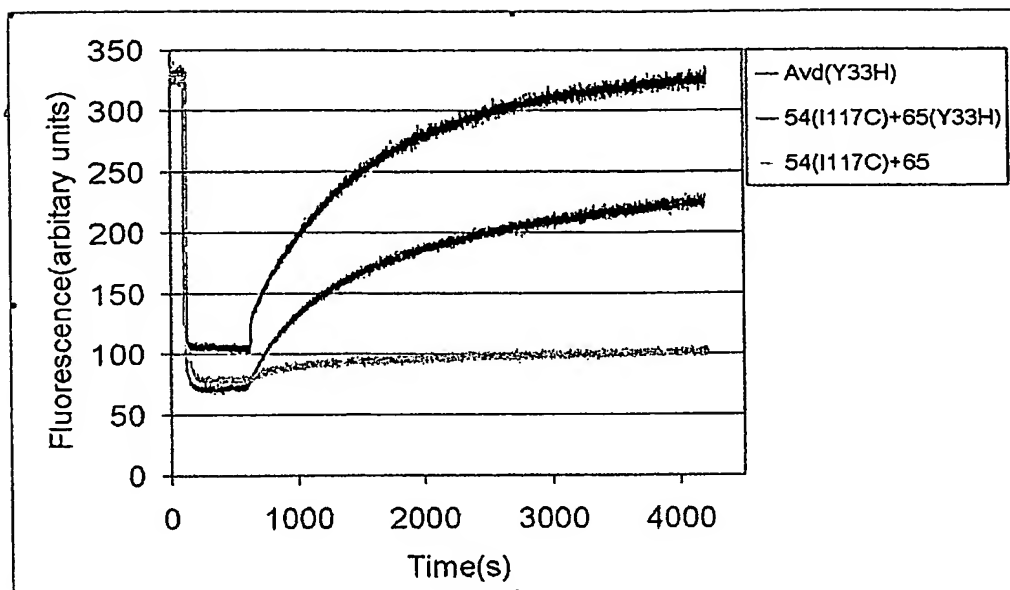


Fig. 6

ATGGTGCACGCAACCTCCCCGCTGCTGCTGCTGCTGCTGCTCAGCCTGGCTCTGGTGGC
TCCCGGCCTCTCTGCCAGGAAGAGGACCCAGCCCACCTTTGGCTTCACCGTCAATTGGA
AGTTTTTCAGAGTCCACCACTGTCTTCACGGGCCAGTGCTTCATAGACAGGAATGGGAA
GGAGGTCTTGAAGACCATGTGGCTGCTGCGGTCAAGTGTTAATGACATTGGTGATGAC
TGGAAAGCTACCAGGGTTCGGCATCAACATCTTCACTCGCCTGCGCACACAGAAGGAGG
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Fig. 7

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LG

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<213> Gallus gallus

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145 150 155 160Lys Ser Gly Gly Ser Thr Thr Val Phe Thr Gly Gln Cys Phe Ile Asp
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Ile Phe Thr Arg Leu Arg Thr Gln Lys Glu Gly Gly Ser Gly Gly Ser
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Asn Met Thr Ile Gly Ala Val Asn Ser Arg Gly Glu Phe Thr Gly Thr
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Tyr Ile Thr Ala Val Thr Ala Thr Ser Asn Glu Ile Lys Glu Ser Pro
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Leu His Gly Thr Gln Asn Thr Ile Asn Lys Arg Thr Gln Pro Thr Phe
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Ser Gly Ser Gly Ser Gly Arg Thr Gln Pro Thr Phe Gly Phe Thr Val
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Asn Trp Lys Phe Ser Glu Ser Thr Thr Val Phe Thr Gly Gln Cys Phe
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